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PHYSIOLOGY OF THE WOOD-ROTTING FUNGI

Contract N6-onr-248, T. O. II,

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GENERAL AND SPECIFIC OBJECTIVES

General Objectives

This research has as its broad objective a fundamental study of the nutrition and physiology of the wood-rotting fungi. These organisms - - classified as Basidiomycetes - - include the so-called "brown rots" and the "white rots" which primarily attack, respectively, the cellulose and the lignin of the cellulose-lignin complex of wood. It is hoped that these studies may contribute to the practical problems of the prevention of wood decay and of the fermentative utilization of carbohydrate materials (including waste cellulose), as well as extending fundamental scientific knowledge of the organisms.

Specific Objectives

More specifically, this investigation involves (1), a systematic study of the nutritional requirements of representative wood-rotting fungi under controlled conditions of artificial culture; and (2), study of various aspects of the physiology of selected organisms. Some 42 species of wood rots - - representative of different types involved in the decay of wood -- are presently under investigation.

Study of the nutritional requirements (1) involves, among other things, development of chemically-defined (synthetic) culture media for growth. This includes the qualitative needs of all the organisms for "trace" elements and inorganic salts, for nitrogen compounds and carbon compounds, and for vitamins or other nutrilites, and the quantitative characterization of these nutrients for the optimal growth of the organisms.

Study of the physiology (2) includes investigation of the cellulolytic enzymes, which are responsible for the primary breakdown of wood;

utilization of various carbohydrates by the organisms; determination of the end-products of the fermentation of carbon compounds, including waste cellulosic materials; study of oxidation-reduction changes in culture and their relation to growth and fermentation; pH and temperature optima, and related problems.

The above studies of nutrition and physiology are fundamental to a rational approach to the control of wood decay and to the practical applications of the organisms in the fermentation of carbohydrate materials to economically valuable products.

BASIC METHODS

The methods used are described in detail in Summary Technical Report #2, January-December, 1949. Briefly, the basic routine method for growing the wood rots was that of aerated liquid culture (submerged culture), using small Erlenmeyer flasks (250-ml.) on a reciprocating shaking machine (shake culture) or, for certain studies, large bottles with forced aeration. Standard inocula and other standard conditions were used throughout. In the nutrition studies, in order to rule out the carry-over of nutrients from one medium to another of different composition, an organism was always serially subcultured in a test nutrient at least three times before growth response was determined quantitatively. Growth was quantitated by the dry weight of mycelial pellets produced in the 70 ml. of culture fluid per flask, at 28°C.

The basal synthetic medium used routinely for qualitatively determining nutrient requirements (particularly nitrogen) is as follows:

BASAL SYNTHETIC MEDIUM

Glucose	10.0 gm./liter
KH_2PO_4 (K=430, P=342 mg./l.)	1.5 gm./liter
$MgSO_4.7H_2O$ (Mg=49.5 mg./l.)	0.5 gm./liter
Thiamine monohydrochloride	1.00 mg./liter
Trace elements	
B (as H_3BO_3)	0.10 mg./liter
Mn(as $MnCl_2.4H_2O$)	0.01 " "
Zn(as $ZnSO_4.7H_2O$)	0.07 " "
Cu(as $CuSO_4.5H_2O$)	0.01 " "
Mo(as $(NH_4)_6Mo_7O_{24}.4H_2O$)	0.01 " "
Fe(as $FeSO_4.7H_2O$)	0.05 " "

To this basal medium the know nitrogen compound under study was added, usually in a concentration of 120 mg./liter total nitrogen.

This basal medium, with an added nitrogen source, supports growth of all of the organisms under study. It was therefore also used as the basis for the development of quantitatively optimal media, varying all constituents in numerous combinations.

BRIEF SUMMARY OF WORK TO DATE

The basic nutritional requirements of 42 representative basidiomycetous wood-rotting fungi have been studied qualitatively and quantitatively in submerged (shake) culture. This work involved approximately 40,000 shake cultures. Most species will produce good to abundant growth in continued serial subculture in a single "standard" synthetic medium containing six "trace" elements, a carbon source (various carbohydrates), a source of nitrogen (a variety of inorganic or organic compounds), thiamine, potassium, phosphorous and magnesium. A few species have additional minimum requirements for other vitamins, for purine or pyrimidine bases, or for special control of Eh or pH. Several of the organisms can utilize one or the other component of the thiamine molecule, instead of requiring the whole molecule. In the nutrition of many of these fungi, biotin can be substituted for thiamine. "Optimal" media, producing up to a few thousandfold increase in growth over the "standard" synthetic medium, have been developed for several of the fungi. The basic studies on nutrition, as originally conceived, are now completed.

Several aspects of the physiology of the wood-rotting fungi have been investigated to a limited extent, some with special reference to their application to the practical problems of the control of wood decay and

to the utilization of carbohydrate materials by fermentation. These include studies on optimum temperature; on pH and Eh in relation to growth and its inhibition; pigment formation; and the production, in shake culture, of fungal polysaccharides and organic acids from carbohydrates. A cellulolytic enzyme has been concentrated from the culture fluid of several of the wood rots. Bark and other celluloses have been shown to be degraded by these organisms in submerged culture.

Specific studies to date may be grouped in the following categories.

1. Growth of wood rots in non-synthetic culture media
2. Development of purely synthetic (chemically-defined) media for growth and nutrition studies
3. Utilization of different forms of organic and inorganic nitrogen
4. Utilization of different carbon compounds
5. Growth curves of the wood rots
6. Vitamin requirements, substitutions, components and synthesis
7. Optimum temperature for growth
8. Optimum pH for growth
9. Oxidation-reduction potential (Eh) in relation to growth
10. Development of synthetic media optimal for growth
11. Separation and concentration of cellulolytic enzymes
12. Development of"cellulase assay tube" for rapid determination of cellulase activity.
13. Production of organic acids by wood-rotting fungi
14. Pigment production
15. Production of fungal polysaccharides

16. Method for determining cellulose breakdown in sawdust
17. Large-scale growth of wood rots in aerated liquid culture
18. Fermentation of sawdust, bark, and other complex carbohydrate materials
19. Respiration studies

PROGRESS

Results since December, 1951.

Because only two graduate students worked on the project half-time from January to June, 1952, the studies were carried on at a lower rate than previously, and were not completed.

1. Studies on oxidative metabolism

Using the Warburg respirometer, a technique has been developed for measuring the oxygen uptake of Polyporus palustris, and determinations have been made of the oxygen uptake of this organism with various carbohydrates.

For consistent results in measuring the oxygen uptake, the inoculum must be properly prepared and standardized. After many trials to obtain a uniform mycelial suspension with a high exogenous respiration, the following procedure was the best that could be devised. From a stock culture of the organism on a potato dextrose agar slant, transfer a small piece of mycelium to 70 ml. of 2% malt extract in a 250-ml. Erlenmeyer flask. Incubate at 28° C. on the shaking machine for 5 days to produce pellets. Transfer the contents of the flask to a Waring blendor and blend the pellets for one minute. Transfer an aliquot of the blended suspension to a 15 ml. centrifuge tube and spin down for two minutes at

2000 r.p.m. Discard the supernatant, resuspend the cells in distilled water, and centrifuge again. Resuspend the cells in distilled water to give a suspension of about 10% by volume. One ml. of this suspension is then used to inoculate 70 ml. of 2% malt extract in a 250-ml. Erlenmeyer flask for starting the final inoculum. Incubate on the shaking machine for 48 hours to obtain the basic inoculum for the Warburg determinations. The pellets may now either be blended in a Waring blender for 5 seconds, or transferred intact to a 50 ml. cetrifuge tube. Unblended pellets give somewhat better results later. In either case centrifuge at 2000 r.p.m. for 2 minutes, wash with distilled water, and repeat three times. After the final centrifugation resuspend the cells in 50 ml. of phosphate buffer, pH 5.5, transfer to a 250-ml. Erlenmeyer flask, and put on the shaking machine for 24 hours to starve the cells in order to decrease endogenous respiration. Centrifuge the cells and resuspend in phosphate buffer to give a suspension of approximately 20% by volume. One ml. of this inoculum is used per flask in the Warburg apparatus.

Using the above standard inoculum, the oxygen uptake of Polyporus palustris was determined manometrically with several carbohydrates in 1% concentration as substrates. The endogenous respiration of the starved cells was measured at the same time, and the exogenous figures corrected for the endogenous value. Table 1 shows the corrected exogenous respiration values, expressed as micro-liters of oxygen per milligram of cells per hour. The values shown are averages of triplicate determinations. (Endogenous values varied between about 1 and 2 micro-liters O_2/mg./hr.)

TABLE 1

Exogenous respiration of P. palustris on various carbohydrates, corrected for endogenous respiration. Oxygen uptake expressed as microliters O_2/mg./hr. Figures are averages of triplicate determinations.

Substrate	Average µl./mg./hr.	Standard Deviation
Fructose	2.29	0.54
Galactose	2.14	0.12
Sucrose	2.13	0.06
Glucose	2.02	0.12
Maltose	1.94	0.16
Mannose	1.92	0.11
Dextrin	1.73	0.12
Xylose	1.67	0.12
Glycogen	1.60	0.14
Arabinose	1.49	0.17
Cellobiose	1.20	0.16
Lactose	0.49	0.05
Ribose	0.43	0.07
Rhamnose	0.16	0.05

The data in Table 1 show that this organism utilizes all of the carbohydrates, although lactose, ribose and rhamnose are used rather poorly. (The oxygen uptake values for this organism are rather low compared with most bacteria, other fungi and other types of cells. Whether this is normal, or whether the best technique has not been developed, is not known.)

2. Vitamin studies.

We have previously reported that 36 of our 42 cultures of wood rots will grow in continued subculture with biotin substituted for thiamin in the basal medium, although the amount of growth in biotin is less than that in thiamin. This suggests that the organisms have two metabolic pathways. Microbiological assays for thiamin have been carried out on the culture filtrates after growth of these organisms, and no thiamin has been found. This is further evidence for two separate metabolic pathways, since in the presence of biotin apparently no thiamin is synthesized, or at least is not present in the culture fluid. Thiamin assays on the mycelium of these organisms were somewhat inconclusive, as a good digest of the mycelium could not be made. The data, such as they are, suggest that a few organisms contain traces of thiamin in the mycelium, but apparently appreciable quantities of thiamin are not produced.

3. Production of sterols.

Preliminary screening studies indicate that some of the wood rots form sterols in malt extract media, and smaller amounts in synthetic media.

4. Chromatographic separation of fungal pigments.

Preliminary work suggests that this method may have merit in separating the pigments which are produced in large quantities by some of our fungi.

5. Preparation of active mycelial extracts.

Cell-free, enzymatically-active mycelial extracts would be useful for numerous enzyme studies. Preliminary attempts to obtain such extracts were inconclusive when mycelial pellets were blended in a mortar with

sand and buffer at room temperature, and the filtrate tested in the Warburg manometer with sodium pyruvate. Absence of oxygen uptake indicated either no carboxylase enzyme in the mycelium, or inactivation of this enzyme by the method of preparation. Further studies, using P. palustris, have yielded a cell-free extract which showed carboxylase activity. Presumably the same technique could be used for obtaining other endo-enzymes.

Mycelial pellets were obtained by growing the organism under forced aeration in two-quart bottles containing 2% malt extract. Incubation was at 28°C. for four days. The pellets were then separated from the medium by pressing through cheesecloth, and the mycelium lyophilized and pulverized. 0.5 grams of the pulverized mycelium were transferred to a 50-ml. centrifuge tube, 10 ml. of phosphate buffer (pH 5.0) added, and the tube shaken for one minute. The suspension was then centrifuged for three minutes at 2000 rpm, and the supernatant decanted for activity studies. The carboxylase activity of the extract was determined by measuring the rate of CO_2 evolution from sodium pyruvate with standard Warburg manometer procedures, at 28°C.

Preliminary studies of the influence of pH on carboxylase activity showed pH5 to be better than pH6. At the former pH, 154.3 microliters of CO_2 per hour were evolved; at the latter pH, 130.8.

To determine whether the cell-free extract contained cocarboxylase as well as carboxylase, runs were made with sodium pyruvate in a concentration of 5 mg./ml. of extract, with the "standard" extract, standard extract plus 2 micrograms cocarboxylase (pure enzyme) per ml., and standard extract plus 5 micrograms cocarboxylase per ml. Microliters of CO_2 per hour were, respectively, 130.8, 128.6 and 129.8, showing that cocarboxylase was

present in the extracted mycelium, and further addition of it to the extract had no appreciable effect.

The effect of the enzyme-substrate concentration on CO_2 evolution was studied at two concentrations of substrate and four concentrations of enzyme, at pH5.0, with no added cocarboxylase. Table 2 shows the cumulative results at 10-minute intervals, of CO_2 evolution expressed as microliters per hour.

TABLE 2

Carboxylase activity of cell-free extract of P. palustris. Effect of enzyme-substrate concentration with sodium pyruvate, at pH5.0, 28°C . Activity expressed as microliters CO_2 per hour, at 10-minute intervals. S= "standard" extract concentration.

Substrate: sodium pyruvate
Concentration: 1 mg./ml. of extract

µl. CO_2/hour

Time in minutes	Concentration of enzyme extract			
	S	0.5 S	0.25 S	0.125 S
10	28.1	21.3	7.95	8.58
20	56.2	37.4	17.5	15.5
30	67.0	42.7	20.7	15.5
40	82.6	55.2	27.0	18.9
50	98.2	67.6	35.0	27.4
60	110.6	74.7	39.7	30.9
70	116.8	83.6	44.5	32.6
80	123.1	88.9	46.1	34.3
90	127.8	94.3	50.9	37.8
100	132.4	97.8	52.5	37.8
110	133.9	103.2	57.2	41.2
120	137.1	108.5	62.0	46.4

Substrate: sodium pyruvate
Concentration: 5 mg./ml. of extract

µl. CO_2/hour

Time in minutes	Concentration of enzyme extract			
	S	0.5 S	0.25 S	0.125 S
10	34.6	18.6	9.34	8.91
20	69.2	40.6	20.2	13.4
30	85.1	48.1	23.3	16.3
40	109.6	64.5	31.1	22.3
50	126.8	80.6	40.4	29.7
60	154.3	92.9	46.7	34.2
70	168.6	100.7	49.8	35.6
80	184.5	111.6	54.5	38.6
90	198.9	120.8	59.1	43.1
100		130.2	62.2	44.6
110		142.6	70.0	49.0
120		151.9	76.2	53.5

The results in Table 2 show that the breakdown of sodium pyruvate by carboxylase is influenced by the concentration of enzyme and substrate.

CONCLUSIONS

This contract on the physiology of the wood-rotting fungi ends--although the research is not completed--as of this report. The studies on nutrition, as originally conceived, were finished. Several new lines of investigation as regards nutrition were opened up, and many fundamental aspects of the physiology of these organisms remain to be investigated. Included in the latter category are various enzyme studies, respiration studies, and production of end products of metabolism. The research to date has yielded results which give a more complete understanding of the nutrition and of some of the metabolic activities of the wood-rotting fungi. Future work, both fundamental and applied, should lead to better control of wood rotting, and perhaps to the utilization of these organisms in fermentation.

The final report, covering four and one-half year's work on this project, will be available sometime this summer.

BIBLIOGRAPHY

No papers have been published during the current report period (January, 1952 - June, 1952) except the mimeographed ONR Semi-annual Progress Report #6, July-December, 1951. January, 1952.

OTHER ASPECTS OF THE STUDIES

a. Changes in emphasis or orientation.

With the completion of the nutrition studies, several aspects of the physiology of the organisms were started but not finished.

b. Personnel changes.

Dr. Richard Henderson, biochemist on theso investigations, left the University as of January 31, 1952. Miss Melva Derrick, who has worked on this contract for three years, received her Doctor's degree in January of this year. Mr. Chester Koda replaced Mr. David Cooman as a half-time technician on February 1, 1952. Miss Josephine D'Agostino, technician, received her Master's degree in June. Mr. Paul Borick and Mr. Harvey Newcomb have worked on various phases of these studies without remuneration.

c. Graduate students on contract.

Miss Josephine D'Agostino and Mr. Chester Koda.

d. Other research support.

The Department of Plant Sciences continues to furnish secretarial assistance, space, and certain capital equipment. The university Institute of Industrial Research has purchased certain pieces of apparatus used in these investigations, and has furnished a full-tuition fellowship for a graduate student (Miss D'Agostino) on this contract for the academic year September, 1951 - June, 1952.

e. Difficulties encountered

None.

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